AD_________________

Award Number: **W81XWH-08-1-0424**

TITLE: **Recruitment & Regulation of N-Wasp by F-Bar Family Member CIP4 in Invasive Breast Cancer** Cells

PRINCIPAL INVESTIGATOR: **Christina S. Pichot**

CONTRACTING ORGANIZATION: **The University of Texas Health Science Center Houston, Texas** 77030

REPORT DATE: July **2009**

TYPE OF REPORT: **Annual Summary Report**

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

☒ Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)* 01-07-2009	**2. REPORT TYPE** Annual Summary	**3. DATES COVERED** *(From - To)* 16 Jun 2008 - 15 Jun 2009
4. TITLE AND SUBTITLE Recruitment & Regulation of N-Wasp by F-Bar Family Member CIP4 in Invasive Breast Cancer Cells		**5a. CONTRACT NUMBER** **5b. GRANT NUMBER** W81XWH-08-1-0424 **5c. PROGRAM ELEMENT NUMBER**
6. AUTHOR(S) Christina S. Pichot Email: christina.s.pichot@uth.tmc.edu		**5d. PROJECT NUMBER** **5e. TASK NUMBER** **5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Texas Health Science Center Houston, Texas 77030		**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)** **11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The focus of this project is the contribution of the Cdc42-interacting protein CIP4 to the invasive phenotype of MDA-MB-231 cancer cells. CIP4 is a member of the F-BAR family of proteins, which interact with or induce plasma membrane curvature through their amphipathic BAR domain. My lab has previously shown that CIP4 interacts with N-WASp, a Cdc42 effector and actin polymerization promoter, which is implicated in the formation of, specialized invasive structures known as invadopodia. Invadopodia are actin-rich protrusions that combine cytoskeletal reorganization with localized degradation of extracellular matrix substrates to mediate cellular invasion. In my preliminary research, I noticed increased CIP4 expression in highly invasive breast cancer cell lines. My research investigates the role of CIP4 in promoting invasion and invadopodia in breast cancer cells *in vitro* through its interaction with N-WASp. The aims of the proposed work were 1) to determine the dynamics of CIP4 binding to N-WASp and their localization to the invadopodia, 2) to determine which domains of CIP4 are required for trafficking and activation of N-WASp, and 3) to examine the necessity of this interaction in the formation / function of invadopodia and cellular invasion.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	13	USAMRMC **19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Introduction

The focus of this project is the contribution of the Cdc42-interacting protein CIP4 to the invasive phenotype of MDA-MB-231 cancer cells. CIP4 is a member of the F-BAR family of proteins, which interact with or induce plasma membrane curvature through their amphipathic BAR domains (1-3). My lab has previously shown that CIP4 interacts with N-WASp (2), a Cdc42 effector and actin polymerization promoter which is implicated in the formation of specialized invasive structures known as invadopodia (4-7). Invadopodia are actin-rich protrusions that combine cytoskeletal reorganization with localized degradation of extracellular matrix substrates to mediate cellular invasion (8, 9). In my preliminary research, I noticed increased CIP4 expression in highly invasive breast cancer cell lines. My research investigates the role of CIP4 in promoting invasion and invadopodia in breast cancer cells *in vitro* through its interaction with N-WASp. The aims of the proposed work were 1) to determine the dynamics of CIP4 binding to N-WASp and their localization to the invadopodia, 2) to determine which domains of CIP4 are required for trafficking and activation of N-WASp, and 3) to examine the necessity of this interaction in the formation / function of invadopodia and cellular invasion.

Body

Task 1: Deepen my knowledge and understanding of breast cancer biology.

In the last year, I have completed seminar courses at MD Anderson to further my understanding of cancer mechanisms: Molecular Mechanisms of Human Cancer and Current Topics of Oncogene Research.

I have presented the research described within this project at the annual conference for the Cancer Biology Program at MD Anderson, as well as numerous departmental seminars.

Task 2:. Determine dynamics of N-WASp – CIP4 binding and localization to invadopodia,

CIP4 localization was studied in unstimulated MDA-MB-231 cells grown over a thin layer of FITC-labeled gelatin. After overnight incubation, invadopodia are identifiable as colocalizations of gelatin degradation and punctate actin. Endogenous CIP4 was imaged through immunofluorescence staining with commercially available antibody (BD monoclonal) against CIP4. I found that punctate CIP4 was also localized to the invadopodia structure (see Appendix: **Figure 1**), specifically at the base of the invadopodia. Although N-WASp has been previously localized to invadopodia (6, 7), immunofluorescence staining of endogenous N-WASp was unreliable with commercially available antibodies, so further studies of N-WASp localization will be performed with a fluorophore-tagged N-WASp construct.

In MDA-MB-231 cells cultured on glass coverslips, we utilized acceptor photobleaching FRET to quantify the interaction between CIP4 and N-WASp in response to EGF stimulation. At sites just inside the plasma membrane, we observed a transient increase in the FRET efficiency, indicating an increase in binding between the two molecules (see Appendix: **Figure 2a**) within 10 seconds of EGF stimulus. This was corroborated by co-immunoprecipitation of endogenous CIP4 and N-WASp (see Appendix: **Figure 2b**).

Task 3: Determine which domains of CIP4 are required for trafficking of N-WASp to sites of invadopodium formation.

In immunofluorescence microscopy of MDA-MB-231 cells, siRNA-mediated CIP4 knockdown did not appear to affect the localization of endogenous N-WASp, however this needs to be verified with fluorescently-tagged N-WASp construct since staining of endogenous N-WASp is uncertain with commercially available antibodies. Co-immunoprecipitation of tagged CIP4 constructs with N-WASp is currently being optimized.

Task 4: Quantify the effects of CIP4 – N-WASp binding in N-WASp activation and invadopodial formation and function.

Because Y256 of N-WASp, a Src-targeted site, is only accessible while N-WASp is in an open, active conformation, I evaluated phosphorylation of this site as a metric for N-WASp activity. CIP4 was siRNA-targeted in MDA-MB-231 cells, treated with EGF, and lysates were blotted with a phospho-specific N-WASp antibody. I found that loss of CIP4 decreased the basal phosphorylation (untreated) of N-WASp at Y256 and abrogated the EGF-induced increase seen

in the control cells (see Appendix: **Figure 3**). This suggests that CIP4 promotes the activation of N-WASp, perhaps by stabilizing the open conformation.

To determine the contribution of CIP4 to the formation and function of invadopodia, MDA-MB-231 cells were treated with either CIP4- or N-WASp-targeted siRNA for 72 hours before culturing on a thin-layer of FITC-gelatin overnight. To identify invadopodia, the cells were stained with TRITC-phalloidin and imaged by confocal microscopy. While CIP4 knockdown did not affect the percentage of cells that formed invadopodia (see Appendix: **Figure 4a**), the average number of invadopodia per cell was significantly reduced (see Appendix: **Figure 4b**). This loss of invadopodia was comparable to the loss seen with N-WASp knockdown. This shift in distribution suggested that the loss of CIP4 might affect the stability or maturation of the invadopodia rather than the initial formation. Therefore, I quantified the area of gelatin degradation per cell as a metric of invadopodia size. Again, loss of CIP4 decreased the degradation of gelatin to a level comparable with the N-WASp knockdown (see Appendix: **Figure 4c**).

The formation of invadopodia has been previously correlated with cellular invasion in vitro (10), so I investigated the effects of CIP4 and N-WASp depletion on the invasiveness of MDA-MB-231 cells through Matrigel-coated Boyden chambers. With two independent CIP4-directed siRNAs, I found a 76-78% decrease in the rate of invasion (see Appendix: **Figure 5a**). Again, this was comparable to the loss of invasion occurring after N-WASp knockdown (78%). Although MDA-MB-231 cells invade spontaneously through Matrigel, they are responsive to EGF as a chemoattractant due to overexpression of EGFR. I found an increase in invasiveness of approximately 2.5-fold with EGF in control cells that was abrogated with CIP4 knockdown (see Appendix: **Figure 5b**). Because the interaction between CIP4 and N-WASp is EGF-dependent (see Appendix: **Figure 3**), this loss may be due to a disruption in the signaling pathway downstream of EGF.

Key Research Accomplishments

- Established the localization of CIP4, the Cdc42-interacting protein, to invadopodia of MDA-MB-231 breast cancer cells

- Using apFRET, demonstrated the dynamic interaction between CIP4 and N-WASp at the plasma membrane in response to EGF

- Demonstrated that CIP4 potentiates phosphorylation (activation) of N-WASp in unstimulated and EGF-stimulated MDA-MB-231 cells

- Determined that CIP4 loss decreases the average number of invadopodia per cell in MDA-MB-231 cells grown over gelatin substrate, comparable to N-WASp depletion

- Determined that CIP4 loss decreases the area of gelatin degradation per cells, comparable to N-WASp depletion

- Confirmed that CIP4 depletion inhibits cellular invasion through Matrigel-coated membranes, comparable to N-WASp loss.

- Established requirement of CIP4 for invasion of MDA-MB-231 cells through Matrigel towards EGF chemoattractant

Reportable Outcomes

Manuscripts

Cdc42 Interacting Protein 4 promotes breast cancer cell invasion and formation of invadopodia through activation of N-WASp. CS Pichot, SM Hartig, D Arvanitis, S Jensen, J Bechill, S Marzouk, G Scita, JA Frost, SJ Corey. Submitted to *Cancer Research.*

Conclusion

The last year of work has focused on establishing a role for CIP4, via its interaction with N-WASp, in the invadopodia of invasive MDA-MB-231 breast cancer cells. CIP4, a Cdc42 interaction protein, is a member of the novel F-BAR family of proteins that are characterized by

I had previously shown that high CIP4 expression correlates with the in vitro invasive potential in a panel of breast cancer cell lines. In the last year of research, I have established that CIP4 localizes to invadopodia of cells actively invading a FITC-gelatin substrate. To determine the kinetics of CIP4's interaction with N-WASp, I used apFRET to quantify the dynamic interaction between CIP4 and N-WASp. I found that their interaction at the plasma membrane was transiently increased in response to EGF and was able to confirm this increased binding between endogenous proteins through coimmunoprecipitation. In addition to increased binding to CIP4, a Src-targeted tyrosine residue of N-WASp is phosphorylated in response to EGF treatment. Using siRNA-targeting of CIP4, I demonstrated that this phosphorylation, which is indicative of N-WASp activation, was blocked in CIP4-depleted cells. This supports the hypothesis that CIP4 potentiates the activation state of N-WASp to promote actin polymerization. Although an interaction between CIP4 and N-WASp has been previously described, the EGF responsive dynamic is a novel result of my research. The effects of CIP4 on the activation state of N-WASp have not previously been demonstrated.

To determine the functional consequences of CIP4 depletion, I studied the formation and function of invadopodia in MDA-MB-231 cells grown over FITC-gelatin substrate. In cells treated with CIP4-targeted siRNA, I observed a significant decrease in the average number of invadopodia per cell. The loss of invadopodia was comparable to that seen with N-WASp depletion. Furthermore, I determined that CIP4 loss decreased the area of gelatin degradation per cells, also comparable to N-WASp depletion. To determine whether this loss of invadopodia affected the cellular invasion of MDA-MB-231 cells, I quantified the rate of invasiveness in CIP4- and N-WASp-depleted cells through Matrigel-coated membranes. Using this assay, I found that CIP4 loss impaired invasion at a rate comparable with N-WASp-depletion. Furthermore, the loss of CIP4 blocks the chemotaxis of cells through Matrigel towards an EGF stimulus. No member of the F-BAR family of proteins has been previously implicated in the formation of invadopodia or invasion of cancer cells.

References

1. P. Aspenstrom, *Curr Biol* **7**, 479 (Jul 1, 1997).
2. S. M. Hartig *et al.*, *J Cell Sci* **122**, 2283 (Jul 1, 2009).
3. A. Shimada *et al.*, *Cell* **129**, 761 (May 18, 2007).
4. V. Desmarais *et al.*, *Cell Motil Cytoskeleton* **66**, 303 (Jun, 2009).
5. M. Fukuoka *et al.*, *J Cell Biol* **152**, 471 (Feb 5, 2001).
6. M. Lorenz, H. Yamaguchi, Y. Wang, R. H. Singer, J. Condeelis, *Current Biology* **14**, 697 (2004).
7. H. Yamaguchi *et al.*, *J. Cell Biol. %R 10.1083/jcb.200407076* **168**, 441 (January 31, 2005, 2005).
8. S. Linder, *Trends Cell Biol* **17**, 107 (Mar, 2007).
9. A. M. Weaver, *Clin Exp Metastasis* **23**, 97 (2006).
10. P. J. Coopman, M. T. Do, E. W. Thompson, S. C. Mueller, *Clin Cancer Res* **4**, 507 (Feb, 1998).

Appendix

Figure 1:

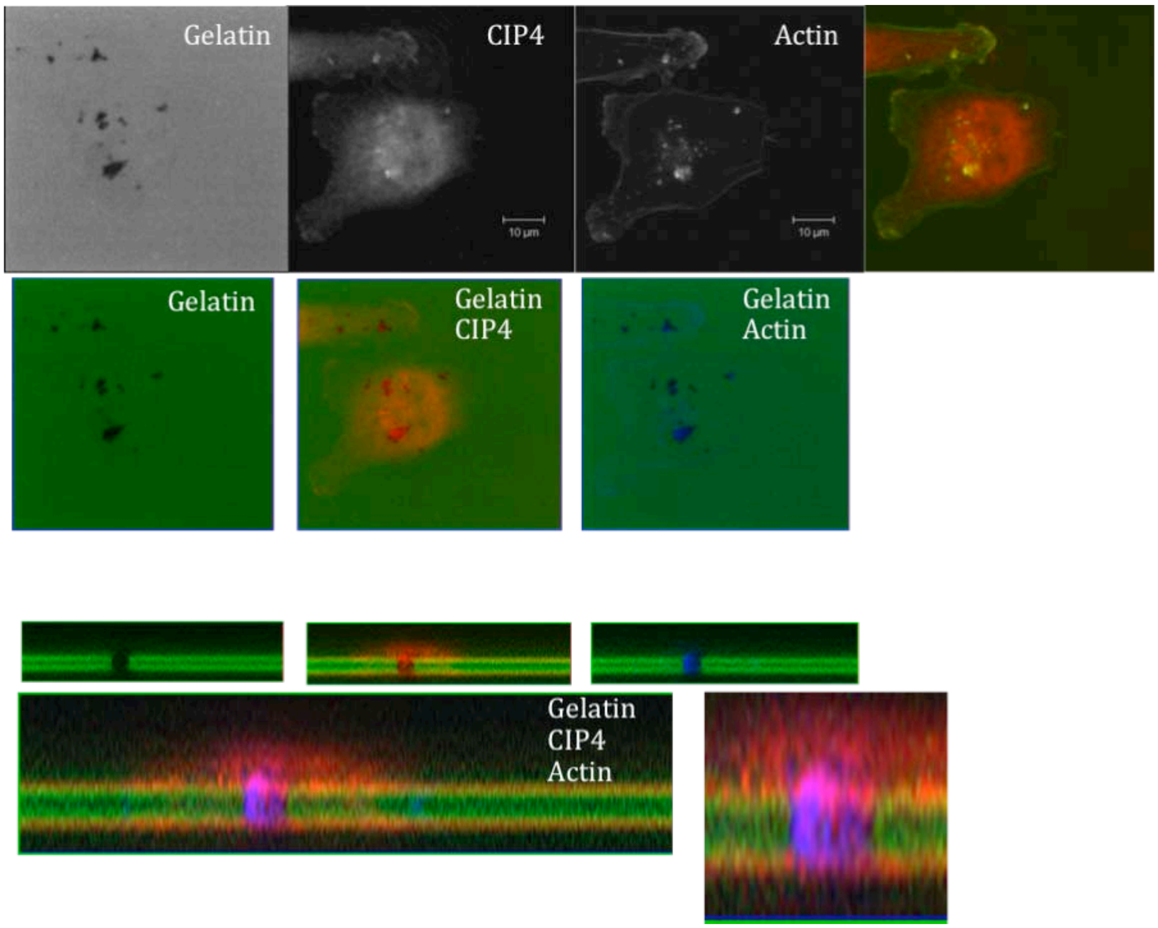


Figure 2:

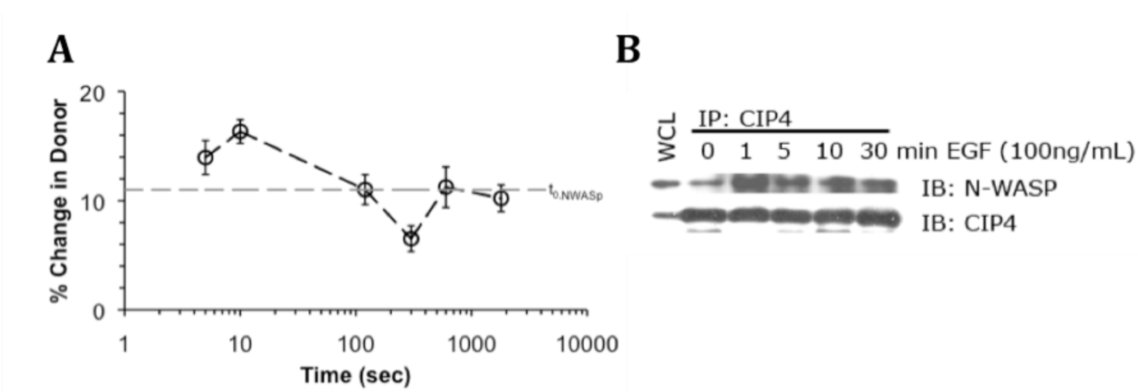


Figure 3:

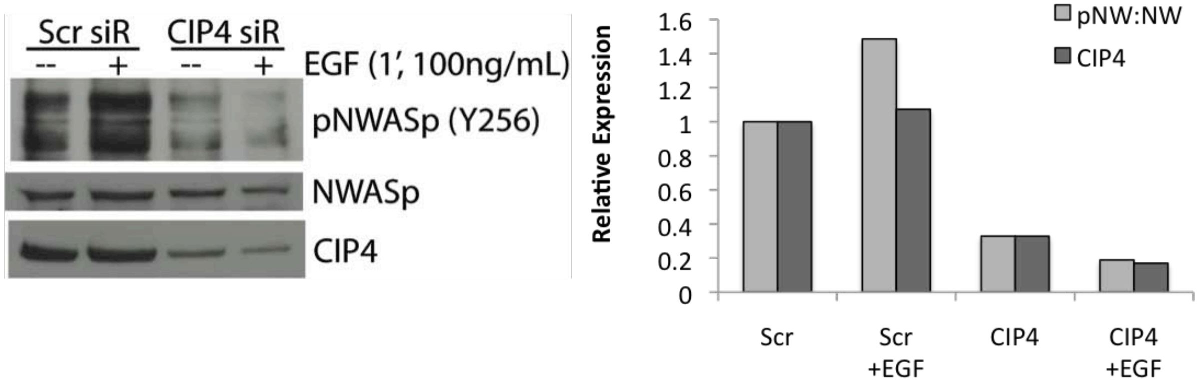


Figure 4:

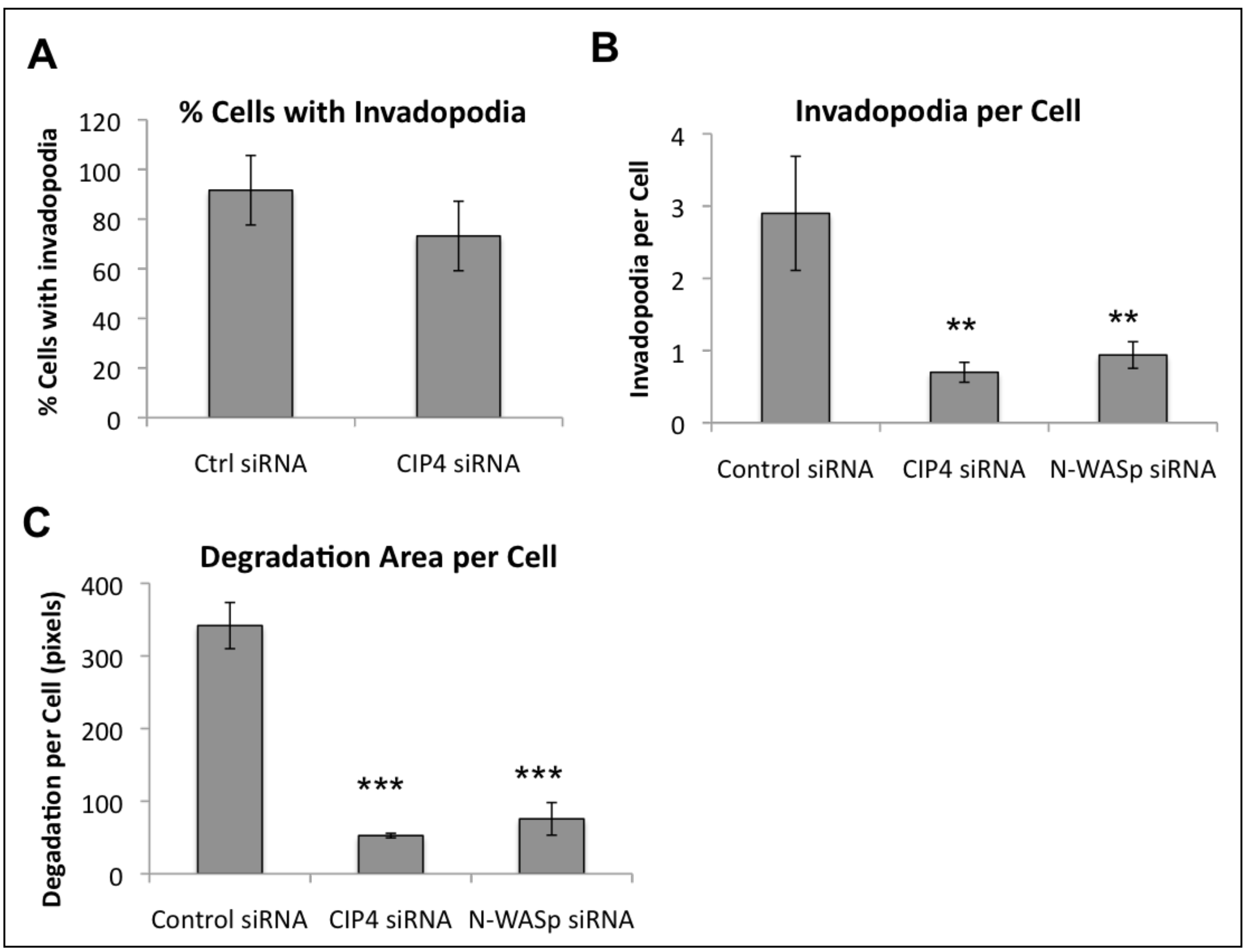
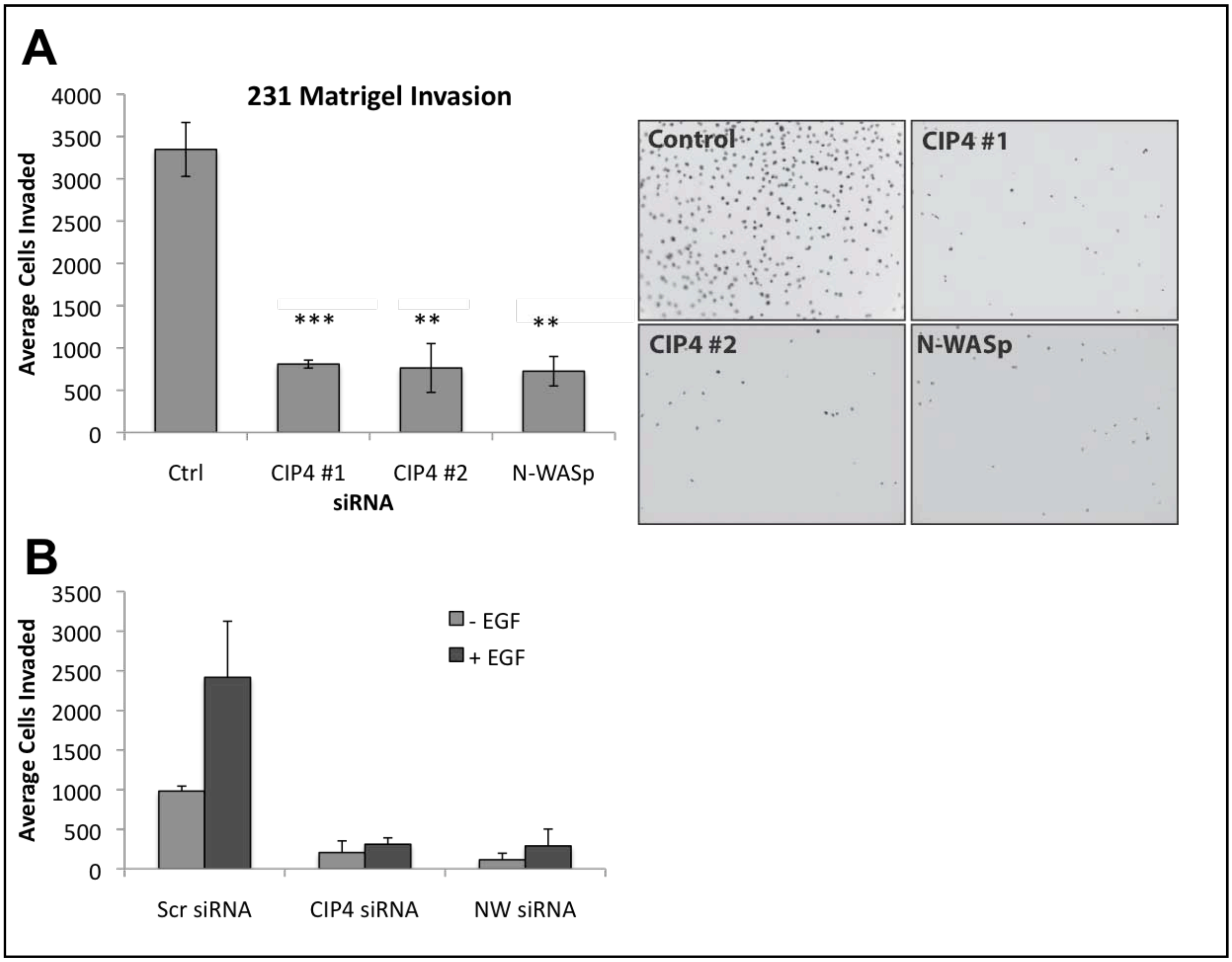


Figure 5:



A
231 Matrigel Invasion
Average Cells Invaded
4000
3500
3000
2500
2000
1500
1000
500
0
***
**
**
Ctrl
CIP4 #1
CIP4 #2
N-WASp
siRNA
Control
CIP4 #1
CIP4 #2
N-WASp
B
Average Cells Invaded
3500
3000
2500
2000
1500
1000
500
0
- EGF
+ EGF
Scr siRNA
CIP4 siRNA
NW siRNA